# Molecular epidemiology of rabies virus isolated of herbivores from Brazilian Amazon

## *Epidemiologia molecular de vírus da raiva isolados de herbívoros procedentes da Amazônia brasileira*

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**Abstract**

Rabies virus samples (n = 17) isolated from bovines (n = 11), equines (n = 4) and buffalo (n = 2) from Pará State (n = 7), Tocantins (n = 6) and Rondônia (n = 4) were submitted to RT-PCR in order to obtain partial sequences of nucleoprotein (N) and glycoprotein gene (G). Nucleotide sequences were analyzed using Neighbor-Joining model, Kimura 2-parameters evolutionary model. All the 17 samples analyzed were related to cluster A, lineage associated with the hematophagous bat *Desmodus rotundus*. The phylogenetic analysis based on the N and G genes, suggests the presence of five sub-lineages (A1-A5), while G gene showed seven sub-lineages (A1-A7). In both phylogenies, sub-lineages A1 to A3 exhibit a similar composition and geographic distribution. Diverse composition of remaining groups of N and G gene is attributable to different sequences used in the alignments for each genomic region. Glycoprotein amino acid sequence showed molecular markers in sub-lineages A2, A3, A4 and A7. This information provides a better comprehension of molecular epidemiology of rabies, starting with the knowledge of viral lineages circulating in the Brazilian Amazon.

**Keywords:** Herbivores. Nucleoprotein. Glycoprotein. Phylogeny. Rabies.

**Resumo**

Amostras do vírus da raiva (n = 17) isoladas de bovinos (n = 11), equinos (n = 4) e bubalinos (n = 2) procedentes do Pará (n = 7), Tocantins (n = 6) e Rondônia (n = 4) foram submetidas à técnica de RT-PCR para amplificação parcial dos genes da Nucleoproteína (N) e Glicoproteína (G). As sequências nucleotídicas obtidas foram analisadas pelo método de reconstrução filogenética Neighbor-Joining com o modelo evolutivo Kimura 2-parâmetros. Todas as 17 amostras pertenceram ao cluster A, que se encontrou na linhagem associado com morcego hematófago *Desmodus rotundus*. A análise filogenética baseada nos genes N e G, sugere a presença de cinco sublinhagens (A1-A5) e sete sublinhagens (A1-A7), respectivamente. Quando se compara ambas as filogenias, as sublinhagens A1 até A3 mostram composição e distribuição geográfica concordante, já a diversidade observada na composição das sublinhagens restantes é atribuída ao uso de sequências de diferentes alinhamentos. A glicoproteína mostrou marcadores moleculares nas sublinhagens A2, A3, A4 e A7, o que fornece elementos para melhor compreensão da epidemiologia molecular da raiva das linhagens circulantes na Amazônia Brasileira.

**Palavras-chave:** Herbívoros. Nucleoproteína. Glicoproteína. Filogenia. Raiva.

## Introduction

Rabies virus (RABV) belongs to Lyssavirus genus, family Rhabdoviridade (RADOSTITS et al., 2000; ACHA; SZYFRES, 2003; FAUQUET et al., 2005), with approximately 200 nm of length and 75 nm of diameter (RUPPRECHT; HANLON; HEMACHUDHA, 2002). In Brazil and Latin America, hematophagous bat *Desmodus rotundus* is the main vector for RABV to livestock (CARNIELI et al., 2009). Epidemiological studies considering molecular diversity and spatial analysis of rabies cases are useful to evaluate surveillance and control disease measures

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(KOBAYASHI et al., 2008; HIRANO et al., 2010). The aim of this study was to characterize genetic RABV isolates from the Pará, Rondônia and Tocantins States and identify molecular markers related to geographical distribution of rabies cases since there is scarce information about livestock rabies in the Brazilian Amazon.

## Materials and Methods

### *Samples*

Seventeen central nervous system (CNS) samples were used, isolates from bovines, equines and buffalo coming from the Pará, Tocantins and Rondônia States between 2004 and 2008. Other sequences retrieved from GenBank were used (Table 1).

Table 1 – Isolates in this study with Genbank accession number, host, year of isolation, city/state and references for each sample – São Paulo – 2013

| Genbank Accession Number (N) | Genbank Accession Number (G) | Host | Year of isolation | City/State | References |
|---|---|---|---|---|---|
| KF005073 | KF005056 | Bovine | 2004 | Cacoal/RO | This article |
| KF005070 | KF005053 | Equine | 2005 | Ponte Alta do Tocantins/TO | This article |
| KF005063 | KF005046 | Bovine | 2005 | Bragança/PA | This article |
| KF005065 | KF005048 | Bovine | 2006 | Santa Maria das Barreiras/PA | This article |
| KF005071 | KF005054 | Bovine | 2006 | São Féliz do Tocantins/TO | This article |
| KF005068 | KF005051 | Bovine | 2006 | Conceição/TO | This article |
| KF005069 | KF005052 | Bovine | 2006 | Ponte Alta do Tocantins/TO | This article |
| KF005061 | KF005044 | Buffalo | 2006 | Quatipuru/PA | This article |
| KF005062 | KF005045 | Buffalo | 2006 | Bragança/PA | This article |
| KF005066 | KF005049 | Bovine | 2007 | Natividade/TO | This article |
| KF005067 | KF005050 | Bovine | 2007 | Combinado/TO | This article |
| KF005074 | KF005057 | Bovine | 2007 | Campo Novo/RO | This article |
| KF005059 | KF005042 | Equine | 2007 | Tracuateua/PA | This article |
| KF005060 | KF005043 | Equine | 2007 | Mãe do rio/PA | This article |
| KF005064 | KF005047 | Bovine | 2007 | Mãe do Rio/PA | This article |
| KF005075 | KF005058 | Bovine | 2008 | Urupá/RO | This article |
| KF005072 | KF005055 | Equine | 2008 | Machadinho do Oeste/RO | This article |
| EF363758.1 | HM486902.1 | *Diphilla ecaudata* | 2004 | São Miguel Tapuio/PI | Castilho et al.(2010b) |
| EF363759.1 | HM486903.1 | *Desmodus rotundus* | 2004 | São Miguel Tapuio/PI | Castilho et al.(2010b) |
| AB297644.1 | - | *Desmodus rotundus* | 2006 | Nova Iguaçu de Góias/GO | Kobayashi et al. (2007) |
| AB297646.1 | - | *Desmodus rotundus* | 2006 | Cocalzinho de Góias/GO | Kobayashi et al. (2007) |
| EF363748.1 | - | Human | 2004 | Portel/PA | Castilho et al. (2010a) |
| EF363750.1 | - | Human | 2004 | Viseu/PA | Castilho et al. (2010a) |
| EF363751.1 | - | Human | 2004 | Viseu/PA | Castilho et al. (2010a) |
| EF363752.1 | - | Human | 2005 | Augusto Corrêa/PA | Castilho et al. (2010a) |
| EF363756.1 | - | Human | 2005 | Augusto Corrêa/PA | Castilho et al. (2010a) |
| - | AB247396.1 | Bovine | 2000 | Nova Olinda/PA | Sato et al. (2006) |
| - | AB247382.1 | Bovine | 2000 | Araguaína/TO | Sato et al. (2006) |
| - | AB247385.1 | Bovine | 2001 | Araguaína/TO | Sato et al. (2006) |
| - | HM486902.1 | *Diphilla ecaudata* | 2004 | São Miguel Tapuio/PI | Castilho et al. (2010b) |
| - | HM486903.1 | *Desmodus rotundus* | 2004 | São Miguel Tapuio/PI | Castilho et al. (2010b) |
| - | AB247384.1 | Bovine | 2000 | Monte Alegre/GO | Sato et al. (2006) |
| - | AB247394.1 | Bovine | 1999 | Alto Taquari/MT | Sato et al. (2004) |
| - | AB110667.1 | Bovine | 1999 | Cáceres/MT | Sato et al. (2004) |
| - | AB110662.1 | Equine | 1998 | Goiás | Sato et al. (2004) |
| - | AB247383.1 | Bovine | 2000 | Mundo Novo/GO | Sato et al. (2006) |
| - | AB519642.1 | *Desmodus rotundus* | 2000 | Águas de Lindóia/SP | Mochizuki et al. (2011) |
| - | AB247391.1 | Bovine | 2004 | Itapecuru/MA | Sato et al. (2006) |
| - | AB247402.1 | Bovine | 2005 | Codó/MA | Sato et al. (2006) |
| - | AB247440.1 | Human | 2005 | Godofredo Viana/MA | Sato et al. (2006) |
| - | AB247404.1 | Bovine | 2005 | Godofredo Viana/MA | Sato et al. (2006) |
| - | AB247399.1 | Bovine | 2004 | Viana/MA | Sato et al. (2006) |
| - | AB247398.1 | Bovine | 2004 | Capinzal do Norte/MA | Sato et al. (2006) |

Rondônia: RO, Tocantins: TO, Pará: PA, Goiás: GO, Piauí: PI, Mato Grosso: MT, São Paulo: SP, Maranhão: MA

### *Fluorescent antibody test (FAT) and mouse inoculation test (MIT)*

All isolates used in this study were diagnosed by FAT test. Samples were submitted to MIT test that allowed virus isolation from field samples (DEAN; ABELSETH; ATANASIU, 1996; KOPROWSKY, 1996). The isolates were analyzed at Laboratório Nacional Agropecuário (LANAGRO) in Pará.

### *RT-PCR and DNA sequencing*

Total RNA was extracted with Trizol® (Invitrogen) according to manufacturer's instructions. Reverse transcription (RT), polymerase chain reaction (PCR) and DNA sequencing were performed using two sets of primers for each gene sequenced. For nucleoprotein gene (N): sense primer (5´TATACTCGAATCATGATGAATGGGGTC GACT3´) and antisense primer 304 (5´TTGAC GAAGATCTTGCTCAT3´). For glycoprotein (G): sense-GA (5´CGCTGCATTTTRTCARAGT3´) and antisense-GB (5´GGAGGGCACCATTTGGTMTC3´) (ORCIARI et al., 2001; SATO et al., 2004). The amplicon produced by the 504/304 pair of primers was 249 nucleotides (nt) long and corresponded to the nucleoprotein gene located between nucleotides 1,286 and 1,533 of the Pasteur Virus strain (accession number M13215.1). The amplicon produced by the GA/GB pair of primers was 917 nt long and corresponded to the glycoprotein gene located between nt 3222 and 4139 of the PV strain. Each PCR product was purified using ExoSAP-IT (GE Healthcare, EUA) and sequenced using ABI PRISM Dye Terminator (Applied Biosystems™, Foster City, CA) kit. Sequences were resolved in a capilar Genetic Analyser 3500 (Applied Biosystems™, Foster City, CA).

After editing, a final 163 nt sequence corresponding to the N gene (nt 1318-1480 of the PV strain) and a final 587 nt sequence corresponding to the gene (nt 3318-3904 of the PV strain) were obtained (Table 1).

### *Phylogenetic analysis and Spatial analysis*

The sequences were analyzed with Phred software available online at (http://as paragin.cenargen.embrapa.br/phph/), aligned with GenBank sequences using CLUSTAL/W application of BioEdit software (HALL, 1999). A neighbor-joining phylogenetic tree was constructed using Kimura 2-parameter evolutionary model with Mega 4.0 (TAMURA et al., 2007) with 1000 bootstrap replicates. Geographic origin of analyzed isolates was represented on maps obtained from IBGE site (http://mapas.ibge. gov.br), and edited with ArcGis version 9.3 software (ESRI, 1996).

### *Polymorphism analysis*

To detect polymorphisms, putative amino acid sequences of nucleoprotein and glycoprotein genes sequences were inferred using BioEdit.

## Results

### *FAT and MIT*

All 17 samples used in this study were positive by FAT or MIT.

### *Phylogenetic analysis and Spatial Analysis*

Figure 1 shows phylogenetic tree obtained based partial sequencing of N gene and spatial analysis respectively. Phylogenetic analysis of N gene revealed three main viral lineages. Lineage A consisted of hematophagous bat-associated strains. Lineage B consisted of dog associated strains. Lineage C was represented by Duvenhage virus (Genbank: EU293119.1) used as outgroup. All Amazon isolates were segregated into hematophagous bat-associated cluster. Lineage A can be separated into five distinct sub-lineages; Sub-lineage A1, composed of isolates from Tocantins related to Southeastern Pará cases; Sub-lineage A2, composed of Tocantins samples and vampire bats sequences from São Miguel Tapuío,

(municipality of Piauí); Sub-lineage A3, consisted of cases from Goiás and Tocantins; Sub-lineage A4, was formed of bovine and human isolates from Rondônia and Portel (Pará); Sub-lineage A5, formed from samples from Northeastern Pará (Viseu and Augusto Corrêa, Municipalities) related to human rabies cases occurring in Pará.

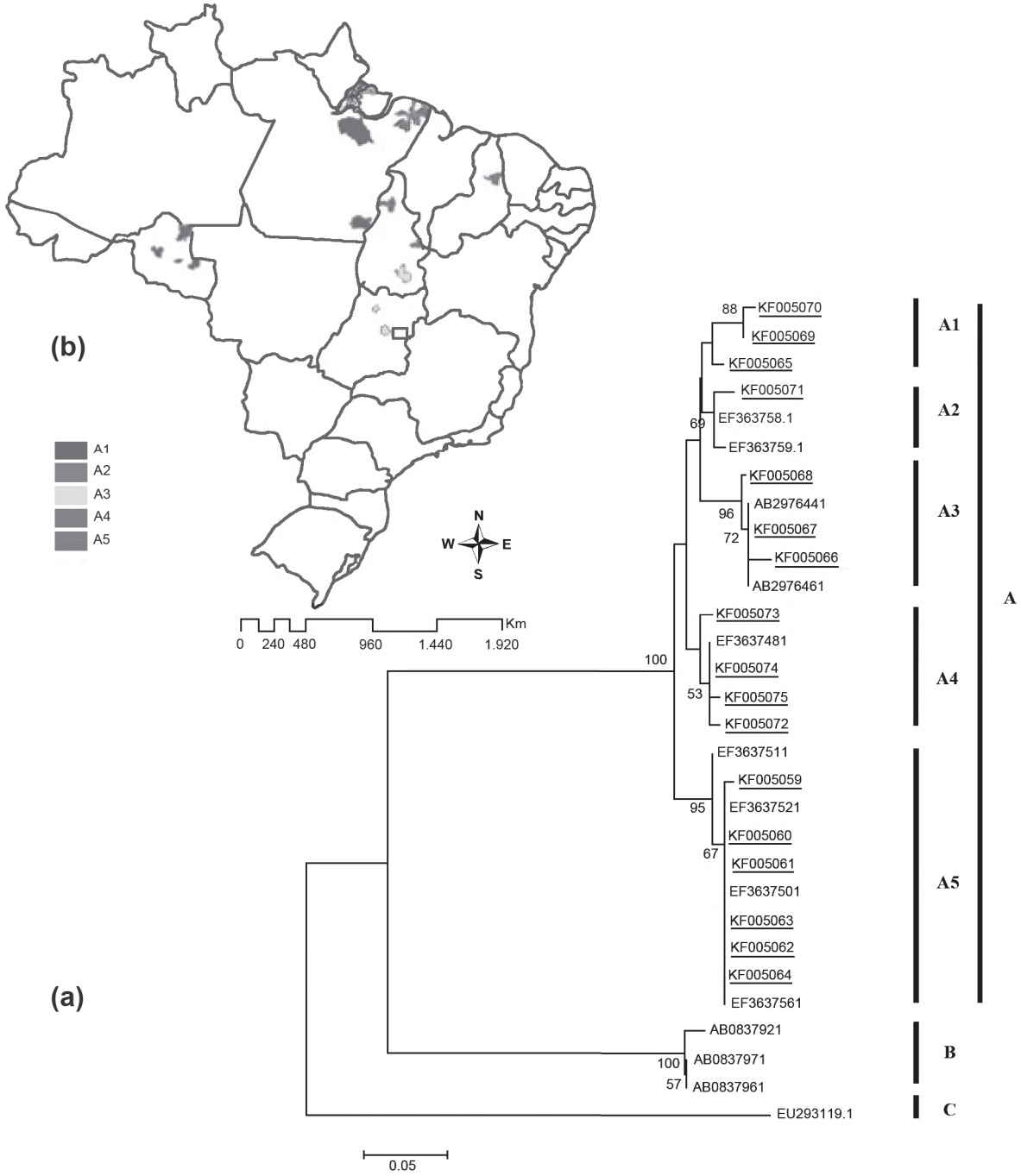


Figure 1 - (a) Phylogenetic tree based on the sequence of 163 nucleotides of the Rabies virus N gene. The phylogenetic analysis was performed by the Neighbor-joining method. The 17 isolates in the present study are shown underlined. Bootstrap values above 50% are shown at the branch node. Equine: EQUI. Bovine: BO, Buffalo: BUF. (b), Geographic distribution of rabies virus strains detected in this study, colors denote phylogenetic groups based on nucleoprotein gene analysis (partial sequences 163 nucleotides of length)
Source: (PEIXOTO, 2014)

Figure 2 shows phylogenetic tree obtained based partial sequencing of G gene and spatial analysis. Phylogenetic analysis based on G gene suggests the presence of three main lineages: Lineage A: vampire bat-related strains, Lineage B: dog-related strains and Lineage C: Duvenhage virus as outgroup. Among Lineage A, seven sub-lineages were identified. Sub-lineage A1, composed exclusively of isolates from Rondônia State. Sub-lineage A2, composed exclusively of isolates from Pará State. Sub-lineage A3, composed exclusively of isolates from Maranhão State. Sub-lineage A4, composed exclusively of isolates from Tocantins State. Sub-lineage A5, one Genbank sequence from Mato Grosso State. Sub-lineage A6, consisted of human isolates from Pará (municipality of Portel) related to Rondônia isolates. Sub-lineage A7 was made up of human isolates from Maranhão and Northeast of Pará.

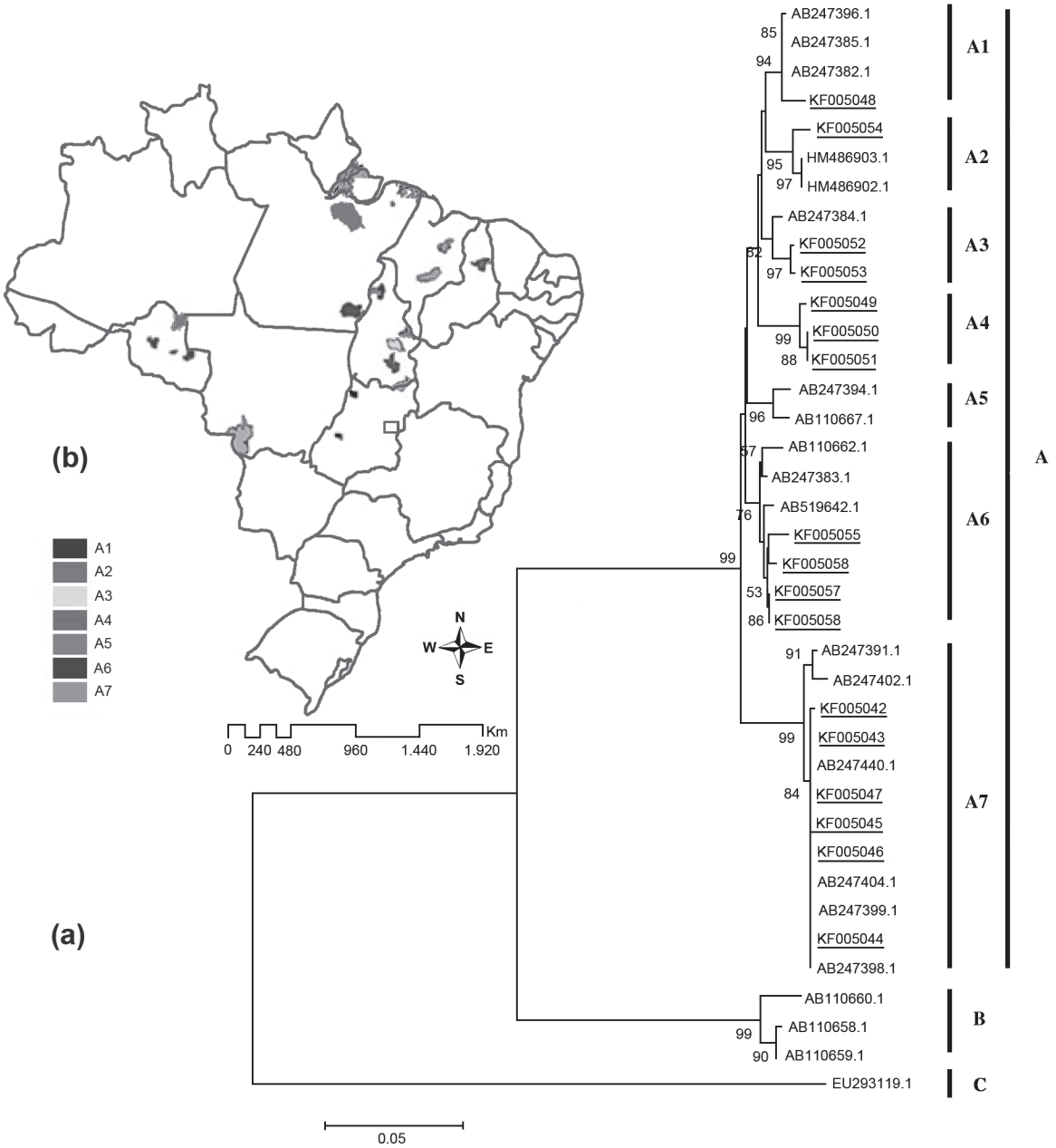


Figure 2 - (a) Phylogenetic tree based on the sequence of 587 nucleotides of the Rabies virus G gene. The phylogenetic analysis was performed by the neighbor joining method. The 17 isolates from the present study are shown underlined. Bootstrap values above 500/o arc shown at the branch node. Equine: EQUI. Bovine: 130V, Buffalo: BUF. (b) Geographic distribution of rabies virus strains detected in this study. Color denotes phylogenetic groups based on glycoprotein gene analysis (partial sequences 587 nucleotides of length)
Source: (PEIXOTO, 2014)

## *Polymorphism analysis*

No molecular markers were identified in the alignment of nucleoprotein for the groups. Molecular markers were identified in the alignment of glycoprotein for the groups: group A2 (Glutamate in position 137), group A3 (Isoleucine in position 6 and Valine in position 109), group A4 (Isoleucine in position 94) e group A7 (Isoleucine in position 48) (Figure 3).

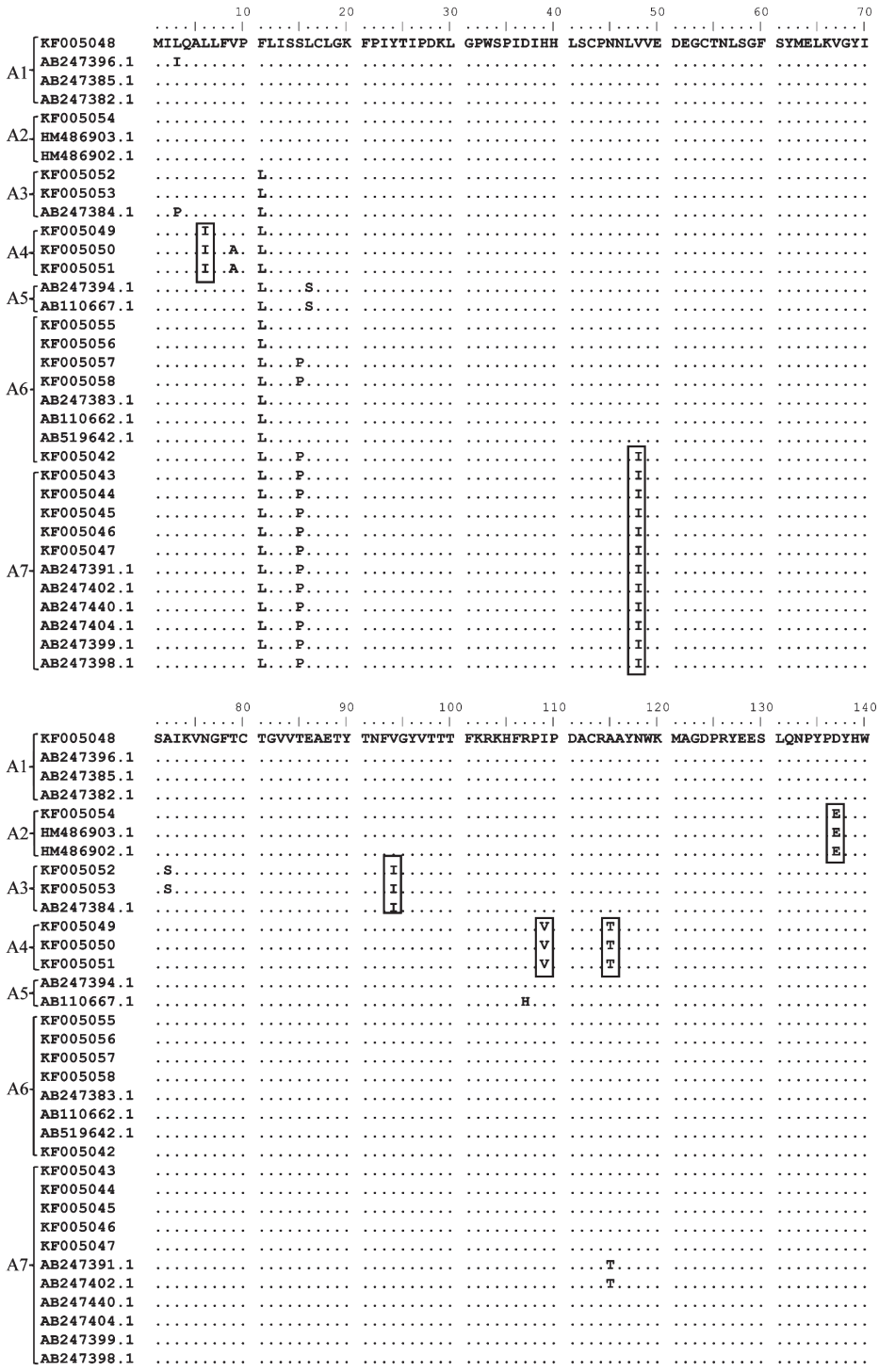


Figure 3 - Alignment of glycoprotein amino acid sequences, showing possible molecular markers from genetic groups
Source: (PEIXOTO, 2014)

## Discussion

Phylogenetic analysis for both genes studied here, revealed two distinct viral lineages circulating in Pará State, one in the Northeast region and the other one in the Southeast. Geographical distance among these areas, probably allowed genetic diversification of the virus. Similar results were obtained by other authors in Pará (TRAVASSOS DA ROSA et al., 2006; BARBOSA et al., 2007). In the phylogeny proposed for both genes, isolates from Northeastern Pará were genetically related to human and herbivorous rabies cases occurring in Maranhão and Pará (SATO et al., 2006; CASTILHO et al., 2010a). This finding could be explained by the geographic proximity of Pará and Maranhão State. There is an intense flow of immigrants and livestock between the North and Northeastern Pará regions, also involving Maranhão State (BARBOSA et al., 2007).

Another factor to consider is cattle transportation to different regions in Northeast Brazil. During the dry season, cattle are transported to save the animals from starvation and drought (GONÇALVES; SÁ-NETO; BRAZIL, 2002).

All 17 sequences studied belong to the lineage usually associated with *D. rotundus.*

The hematophagous bats live in relatively stable colonies, often visiting closer shelters, promoting an indirect communication between other colonies. This aspect of *D. rotundus* ecology, promotes the spread of rabies virus, facilitating contact among infected and susceptible individuals in a colony (LORD, 1988; MCCOLL; TORDO; SETIÉN, 2000).

A possible phylogenetic link between isolates from Rondônia and other distant geographical regions still needs clarification. A hypothesis based only on *D. rotundus* displacement patterns cannot explain this genetic similarity because, according to studies on hematophagous bat biology, the maximum distance they are able to cover is 20 km (GREENHALL; SCHMIDT, 1988; CRESPO et al., 1961; MEDINA et al., 2007), and distances observed in this study are superior to their radius of movement.

An additional factor in this equation is the overlapping livestock circuits of Pará and Rondônia States. We propose that the dissemination pattern of RABV in herbivores of the Brazilian Amazon could be strongly influenced by this circuit overlap (BRASIL, 2006).

In the Tocantins State, the phylogenetic analysis of G gene suggested four distinct circulating lineages. Furthermore, these lineages were supported by molecular markers and exhibited a clear-cut geographic pattern distributed according to municipalities.

Some researchers, who analyzed isolates of cattle from Tocantins State, suggested that topographic features and other ecological aspects strongly influence bat ecology and therefore affect RABV dissemination patterns (KOBAYASHI et al., 2006; KOBAYASHI et al., 2008).

## Conclusions

The topology of the phylogenetic tree based on N and G genes partial sequences from herbivores in the Brazilian Amazon, suggests that all isolates belong to *Desmodus rotundus* strain. Isolates from the same geographic area tend to make a cluster and sublineage division suggests an apparent correlation of these strains with specific geographic areas. In addition, this study revealed molecular markers for different geographic regions, promoting a better understanding of rabies molecular epidemiology in the Brazilian Amazon.

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